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Serum Lipoprotein Pattern in Rats, Dogs and Monkeys, Including Method Comparison and Influence of Menstrual Cycle in Monkeys

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Summary: The serum lipoprotein pattern (HDL-, LDL- and VLDL-cholesterol) was determined in normal untreated rats, dogs and monkeys using the density gradient centrifugation method, and compared with that in humans. The serum of rats (Wistar) and dogs (Beagle) was found to contain mainly HDL-cholesterol, whereas the serum of monkeys (*Macaca fascicularis*) consists of similar amounts of HDL-cholesterol and LDL-cholesterol, as in humans. In contrast to rats and dogs, monkeys showed a very low amount of serum VLDL-cholesterol. Comparison of four different methods (density gradient centrifugation, FPLC®, agarose gel electrophoresis and precipitation methods) for determination of HDL- and LDL-cholesterol in monkey serum showed that density gradient centrifugation and FPLC®-methods have the highest accuracy and recovery rate. The FPLC®-method offers the extra advantages that it requires less sample volume and is less laborious. In a separate experiment, the influence of the menstrual cycle on the serum lipoprotein pattern was investigated in female monkeys. No changes in total cholesterol, HDL- or LDL-cholesterol were detected in serum samples obtained in the follicular and luteal phases of the cycle. In conclusion, monkeys (*Macaca fascicularis*) may be more suitable than rats or dogs for the comparison of the effect of different compounds on serum lipoproteins in pharmacological and toxicological studies, because the lipoprotein pattern in this species is similar to that of humans.

Introduction

Human serum lipoproteins are defined according to their hydrated density (d) and divided into the following classes: Very-low-density-lipoproteins (VLDL; $d < 1.006$ kg/l), low-density-lipoproteins (LDL; $d = 1.006-1.063$ kg/l) and high-density-lipoproteins (HDL; $d = 1.063-1.21$ kg/l). HDL and LDL are the major carriers of cholesterol in human serum. In epidemiological studies, low serum concentrations of HDL-cholesterol and high serum concentrations of LDL-cholesterol were associated with an increased risk of coronary heart disease (1, 2). Moreover, it is known that several compounds such as exogenous and endogenous sex hormones may affect the serum concentration of HDL- and LDL-cholesterol in humans (3).

The structure and composition of serum lipoproteins in various animal species have been investigated in numerous studies. The available data on animal serum lipoproteins were extensively reviewed by *Chapman*, 1980 and 1986 (4, 5) and *Caroll & Feldman*, 1989 (6). Furthermore, in some pharmacological and toxicological investigations, laboratory animals such as rats (7–9), dogs (10, 11) and monkeys (10, 12) have been used for evaluation of the effects of different compounds on their serum lipoprotein pattern. However, in all these studies a variety of methods were used for separation of the lipoprotein fractions, so that the reported lipoprotein patterns might not be directly comparable.

There are no literature reports of the systematic investigation of serum lipoprotein patterns of animals

by density gradient centrifugation and their comparison with the pattern in humans. Therefore, one object of this study was to examine the normal serum lipoprotein pattern (HDL-, LDL-, and VLDL-cholesterol) of rats, dogs and monkeys using density gradient centrifugation under constant conditions, and to compare it with that in humans.

In a second experiment, four different methods commonly used for the determination of human HDL- and LDL-cholesterol were compared in the serum of monkeys, which has a lipoprotein similar to that of human serum. The purpose of this was to check whether the time-consuming and expensive centrifugation method can be replaced by other less laborious methods. Such a comparison was considered to be important as no report is available in the literature concerning the comparison of various methods for HDL/LDL-cholesterol determination using the same animal samples.

Furthermore, the influence of the menstrual cycle on the serum lipoprotein pattern in female monkeys (*Macaca fascicularis*) was also investigated, because a decrease in serum total cholesterol and LDL-cholesterol has been reported in the luteal phase in premenopausal women (13), whereas other reports suggest that no such changes take place during the menstrual cycle (14, 15). This part of the study was performed to evaluate whether female monkeys can be utilised as test species for the characterisation of the effect of different compounds, including sex steroids, on lipid and lipoprotein metabolism.

Materials and Methods

Female Wistar rats (137–165 g, n = 25), Beagle dogs (3–4 years old, n = 2) and *Macaca fascicularis* monkeys (2,5–4,3 kg, n = 20) were used for the studies. The animals were housed in individual cages under conventional conditions and were fasted for at least 16 hours prior to blood collection. Blood was drawn from the v. jugularis of rats and dogs and from the v. antebrachii of monkeys. The serum lipoprotein pattern (HDL, LDL- and VLDL-cholesterol) of rats, dogs and monkeys was evaluated in pooled serum samples using the density-gradient-centrifugation-method. For comparison with humans, one human serum sample was also analysed with this method under similar conditions. Furthermore, four different methods for the determination of HDL- and LDL-cholesterol (density gradient centrifugation, FPLC®, agarose gel electrophoresis and precipitation methods) were compared in serum samples of five monkeys.

Density gradient centrifugation was performed using a modification of the method described by *Redgrave* et al. (16). Serum samples (2 ml) were applied to a five step KBr gradient (d = 1.006, 1.019, 1.063, 1.125 and 1.21 kg/l) consisting of 2 ml each in polyallomer tubes (13.2 ml) and centrifuged in a swinging bucket rotor (24 hours at 20 °C and 288 000 *g*). After centrifugation, 24 fractions of 0.5 ml were collected. Total serum cholesterol and cholesterol concentrations in each fraction were measured with an enzymatic assay using the CHOD-PAP-method of Boehringer Mannheim with the automatic analyser Hitachi 705. In addition, the density of each fraction was determined gravimetrically.

Fast Protein Liquid Chromatography (FPLC®) was performed according to *März* et al. (17). Serum (20 µl) was applied to a 300 mm column of Superose 6 (Pharmacia-LKB) equilibrated with 100 mmol/l Na_2HPO_4, pH 7.4, 200 mmol/l NaCl with a flow rate of 0.3 ml/min. On-line detection was performed photometrically (500 nm) after post-column derivatization with a commercial cholesterol reagent (CHOD-PAP, Boehringer Mannheim) in a 'knitted' capillary. The absolute amounts of HDL- and LDL-cholesterol were calculated on the basis of relative peaks and total cholesterol.

Furthermore, HDL- and LDL-cholesterol were measured in serum by agarose gel electrophoresis using the Hydragel HDL-cholesterol Kit (No. 4006, Sebiachem), and by precipitation methods using commercial kits (Nos. 543004 and 726290, Boehringer Mannheim).

In separate experiment, the influence of the menstrual cycle on the serum lipoprotein pattern was investigated in twelve selected *Macaca fascicularis* monkeys (2.4–4.2 kg) which had a regular cycle length of 26 to 35 days. Blood samples were collected every fourth day after start of bleeding until the onset of the next menstrual flow. All samples were determined for their total cholesterol content as well as their serum lipoprotein pattern using the FPLC®-method. It is known that midcycle elevations of serum luteinizing hormone and follicle-stimulating hormone occur at a mean interval of 11.4 ± 1.1 days after the onset of menstrual flow, and that the luteal phase occurs on average 15.7 ± 1.2 days after the peaks of serum luteinizing hormone and follicle-stimulating hormone in regular cycling *Macaca fascicularis* monkeys (18). The follicular phase lies between the onset of bleeding and elevations of serum luteinizing hormone and follicle-stimulating hormone, and the luteal phase begins after the elevation of these gonadotropins and lasts till the end of the cycle. Day 4 of the menstrual cycle and the last blood sampling day before the next menstrual bleeding were therefore chosen as representative of the follicular and late luteal phases, respectively. The results were statistically evaluated for differences between day 4 and days 24 to 32, using the signed *Wilcoxon*-Test at a level of $\alpha = 0.05$.

Results

The lipoprotein patterns (HDL-, LDL- and VLDL-cholesterol) in the serum of rats, dogs, monkeys and humans using the density gradient centrifugation method are shown in figure 1. The yield of total serum cholesterol for all species was in the range of 97–102% on the basis of cholesterol concentration in each fraction collected.

As is evident from figure 1, rat and dog sera contain mainly HDL-cholesterol with only low amounts of LDL-cholesterol and almost no VLDL-cholesterol. However, a good separation of the lipoprotein fractions of rat serum could not be achieved even using the density gradient centrifugation method. In the monkey serum two distinct peaks of HDL- and LDL-cholesterol and a very small amount of VLDL-cholesterol were detected. As can be seen, the serum lipoprotein pattern in monkeys is similar to that observed in humans, whereas those of rats and dogs are considerably different.

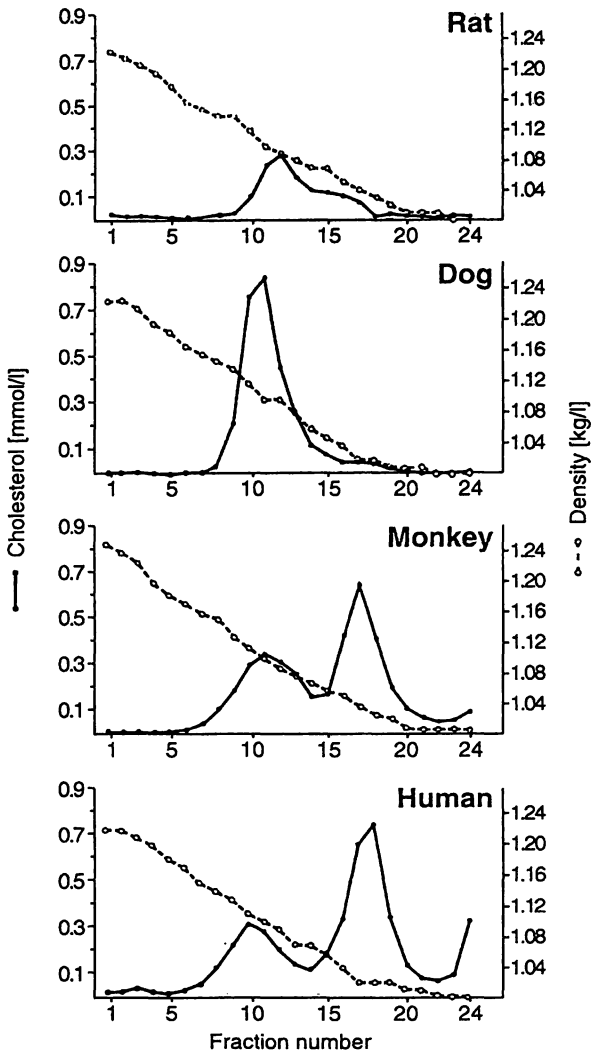

Fig. 1. Serum lipoprotein pattern (HDL- and LDL-cholesterol) in rats, dogs, monkeys and humans determined by density gradient centrifugation. The total cholesterol concentrations of the serum samples were 1.53 (rat), 3.06 (dog), 4.17 (monkey) and 4.64 (human) mmol/l.

For comparison of different methods, density gradient centrifugation, FPLC®, agarose gel electrophoresis and precipitation methods were applied for the determination of HDL- and LDL-cholesterol in the serum of monkeys. Since the low amount of VLDL-cholesterol in monkey serum could only be detected by density gradient centrifugation and FPLC®-methods, this quantity was excluded from the method comparison. Precision of the methods used was checked by measuring 6–10 samples of pooled monkey serum. The coefficients of variation for HDL- and LDL-cholesterol were 1.6 and 1.8% (density gradient centrifugation), 2.6 and 1.8% (FPLC®), 4.3 and 4.9% (agarose gel electrophoresis) and 4.7 and 1.7% (precipitation methods), respectively. The HDL- and LDL-cholesterol in monkey serum were stable for at least 12 weeks in all the methods used, when stored at −20 °C.

The results of HDL- and LDL-cholesterol determination in the serum of monkeys (n = 5) by the four different methods are presented in table 1.

The density gradient centrifugation method was applied as the reference method, since serum lipoproteins were originally classified according to their hydrated density. A very good correlation was found between FPLC® and the density gradient centrifugation method with respect to the absolute values, as well as the relative quantities of HDL- and LDL-cholesterol. Agarose gel electrophoresis showed slightly higher HDL-cholesterol values and lower LDL-cholesterol values than the reference method. The absolute values for HDL- and LDL-cholesterol determined by precipitation methods were lower than those obtained by density gradient centrifugation, FPLC® and electrophoresis. Agarose gel electrophoresis and precipitation methods showed a close correlation in the determination of the relative percentages of HDL- and LDL-cholesterol, despite the fact that they did not agree with density gradient centrifugation and FPLC®-methods, in the determination of absolute values.

Tab. 1. HDL- and LDL-cholesterol in serum samples of monkeys determined by the four different methods (mean value/standard deviation, n = 5)

Constituent	Methods							
	Density gradient centrifugation		FPLC®		Agarose gel electrophoresis		Precipitation (HDL, LDL)	
	[mmol/l]	[%][1]	[mmol/l]	[%][1]	[mmol/l][2]	[%]	[mmol/l]	[%][1]
HDL-cholesterol	1.84 ± 0.16	46 ± 8	1.84 ± 0.26	45 ± 9	2.23 ± 0.31	53 ± 11	1.53 ± 0.31	52 ± 12
LDL-cholesterol	2.28 ± 0.75	54 ± 8	2.36 ± 0.75	55 ± 9	2.02 ± 0.78	47 ± 11	1.45 ± 0.52	48 ± 12

[1] calculated on the basis of Σ HDL- and LDL-cholesterol (mmol/l) set at 100%
[2] calculated on the basis of total cholesterol (4.27 ± 0.70 mmol/l) and percent distribution of HDL/LDL

In a separate experiment, the influence of the menstrual cycle on the serum lipoprotein pattern in monkeys was investigated. Analysis by FPLC® of samples collected from twelve monkeys every fourth day during the cycle showed no alterations of serum total cholesterol, HDL-, LDL- and VLDL-cholesterol during the cycle. The results of the lipoprotein pattern obtained with serum samples from day 4 (follicular phase) and days 24 to 32 (late luteal phase) using the FPLC®-method are presented in table 2. No statistically significant differences were observed in serum total cholesterol, HDL-, LDL- or VLDL-cholesterol between the two cycle phases using the *Wilcoxon*-test.

Discussion

The results obtained in this study demonstrate that the serum of rats (Wistar) and dogs (Beagle) contains mainly HDL-cholesterol and only low amounts of LDL-cholesterol and almost no VLDL-cholesterol. In contrast to these two animal species, monkey serum contains approximately similar amounts of HDL- and LDL-cholesterol and a very low amount of VLDL-cholesterol. Thus, the serum lipoprotein pattern of monkeys is similar to that of human serum as described in the literature (19).

Furthermore, four different methods (density gradient centrifugation, FPLC®, agarose gel electrophoresis and precipitation methods) for determination of HDL- and LDL-cholesterol in serum of monkeys were compared. The method comparison was performed with monkey serum because the serum lipoprotein pattern in this species, in contrast to those of rats and dogs, is similar to that of humans. In addition, a comparison of methods using serum of rats and dogs was not considered to be appropriate, in view of the limit of sensitivity of various methods for detection of different lipoprotein fractions.

Comparison of methods for determination of HDL- and LDL-cholesterol in monkey serum showed a very good correlation between FPLC® and the reference method (density gradient centrifugation). Agarose gel electrophoresis, however, showed slight to moderate differences for the absolute values of HDL- and LDL-cholesterol. These variations are probably due to the staining procedure of this method which, in addition, is not sensitive enough to detect the very low amount of VLDL-cholesterol in monkey serum.

Precipitation methods showed a somewhat low recovery for HDL and an even lower recovery for LDL-cholesterol. Since the test kits used for these precipitation methods are optimised for human serum, the low recovery of the precipitation methods for HDL- and LDL-cholesterol in monkeys is most probably due to the precipitation properties of HDL and LDL in this species, which might be different from those of humans.

As determined by all methods, the HDL- and LDL-cholesterol of monkey serum was stable for at least 12 weeks at −20 °C.

No changes in total cholesterol, HDL-, LDL- or VLDL-cholesterol were found throughout the menstrual cycle of female monkeys. This was also demonstrated statistically by comparison of the values on representative days of the follicular and luteal phases. In humans, several studies on the influence of the menstrual cycle on serum lipids and lipoproteins have been published with differing results. *Kim & Kalkhoff* (13) detected a slight decrease of total cholesterol and LDL-cholesterol in serum in the luteal as well as late luteal phase when compared with the follicular phase of the menstrual cycle. In contrast to this study, more recent investigations by *Lebech* et al. (14) and *Brockerhoff* et al. (15) showed no changes in lipids and lipoproteins throughout the cycle. *Lebech* et al.

Tab. 2. The influence of menstrual cycle on the serum total cholesterol, HDL-, LDL- and VLDL-cholesterol in monkeys (mean value/standard deviation, n = 12)

Day after the onset of menstrual flow	Total serum cholesterol (mmol/l)	Fast protein liquid chromatography (FPLC®)					
		Relative distribution (%)			Absolute values (mmol/l)[2]		
		HDL-cholesterol	LDL-cholesterol	VLDL-cholesterol	HDL-cholesterol	LDL-cholesterol	VLDL-cholesterol
Day 4 (follicular phase)	4.23 ± 0.67	49.4 ± 7.0	48.7 ± 6.7	2.0 ± 0.7	2.09 ± 0.48	2.06 ± 0.43	0.08 ± 0.03
Days 24–32[1] (luteal phase)	4.16 ± 0.98	50.4 ± 8.7	47.6 ± 8.4	1.9 ± 1.1	2.11 ± 0.72	1.98 ± 0.54	0.08 ± 0.04

[1] last blood sampling day prior to the onset of the next menstrual flow
[2] calculated on the basis of total serum cholesterol and percent distribution of the respective fractions

(14) assumed that the changes in sex hormone levels during the menstrual cycle in women are of too short a duration to affect the liver's metabolism of lipoproteins. This may also apply to monkeys, as indicated by the present results in female *Macaca fascicularis*.

In conclusion, these results demonstrate that in order to compare the effect of different compounds on serum lipoproteins in experimental animals and humans, monkeys (*Macaca fascicularis*) may be more suitable than rats or dogs, since the lipoprotein pattern in this species is similar to that of humans. The results of the density gradient centrifugation and FPLC®-methods for the evaluation of HDL- and LDL-cholesterol in monkey serum correlated well and they show a greater accuracy and recovery rate than the other methods. However, the FPLC®-method offers the additional advantages that it requires a smaller sample volume and is less laborious. For characterisation of the effect of different compounds on lipid metabolism in female monkeys, the day of blood sampling seems to be of minor importance. Thus, no significant differences in the serum lipoprotein cholesterol pattern were detected at various timepoints, including the follicular and the late luteal phases of the menstrual cycle.

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